PATENT COOPERATION TREATY

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

From the INTERNATIONAL BUREAU

To:

HWANG, E-Nam
Yegun Building
3rd floor
823-42 Yoksam-dong
Kangnam-ku
Seoul 135-080
RÉPUBLIQUE DE CORÉE

Date of mailing (day/month/year)
25 August 2000 (25.08.00)

Applicant's or agent's file reference
PCT K013

IMPORTANT NOTIFICATION

International application No.
PCT/KR00/00026

International filing date (day/month/year)
14 January 2000 (14.01.00)

1. The following indications appeared on record concerning:

[X] the applicant [ ] the inventor [ ] the agent [ ] the common representative

Name and Address
DAE SANG CORPORATION
52-1 Kayang-dong
Kangseo-ku
Seoul 157-200
Republic of Korea

State of Nationality: KR
State of Residence: KR
Telephone No.:
Facsimile No.:
Teleprinter No.:

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[X] the person [ ] the name [ ] the address [ ] the nationality [ ] the residence

Name and Address
KANG, Yong, Ho
Yongnam University
Applied Microbiology Dept.
712-749 Dae-dong
Kyongsan-city
Kyongsangbuk-do
Republic of Korea

State of Nationality: KR
State of Residence: KR
Telephone No.:
Facsimile No.:
Teleprinter No.:

3. Further observations, if necessary:
The person in Box 2 is now the only applicant and inventor for all designated States.

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[X] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No.: (41-22) 740.14.35

Authorized officer
Ting Zhao
Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing: 20 July 2000 (20.07.00)	
International application No.: PCT/KR00/00026	Applicant's or agent's file reference: PCT K013
International filing date: 14 January 2000 (14.01.00)	Priority date: 14 January 1999 (14.01.99)
Applicant: KANG, Yong, Ho	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International preliminary Examining Authority on:

10 May 2000 (10.05.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland	Authorized officer: J. Zahra
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

COPY FOR IB

PATENT COOPERATION TREATY

REC'D 08 JUN 2001

WIPO PCT

14

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Artcle 36 and Rule 70)

Applicant's or agent's file reference PCT13	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. **PCT/KR00/00026**	International filing date *(day/month/year)* 14 JANUARY 2000 (14.01.2000)	Priority date *(day/month/year)* 14 JANUARY 1999 (14.01.1999)

International Patent Classification (IPC) or national classification and IPC

IPC7 C12N 15/53, C12N 15/70, C12N 15/62

Applicant

Kang, Yong Ho

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 4 sheets, including this cover sheet.

☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of _____ sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☐ Certain observations on the international application

Date of submission of the demamd	Date of completion of this report
10 MAY 2000 (10.05.2000)	30 MAY 2001 (30.05.2001)

Name and mailing address of the IPEA/KR	Authorized officer
Korean Intellectual Property Office Government Complex-Daejeon, Dunsan-dong, Seo-gu, Daejeon Metropolitan City 302-701, Republic of Korea	LIM, Hea Joon
Facsimile No. 82-42-472-7140	Telephone No. 82-42-481-5590

Form PCT/IPEA/409 (cover sheet) (July 1998)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International aplication No.
PCT/KR00/00026

I. Basis of the report

1. With regard to the elements of the international application:*

[X] the international application as originally filed

[ ] the description:
pages _____ , as originally filed
pages _____ , filed with the demand
pages _____ , filed with the letter of _____

[ ] the claims:
pages _____ , as originally filed
pages _____ , as amended (together with any statment) under Article 19
pages _____ , filed with the demand
pages _____ , filed with the letter of _____

[ ] the drawings:
pages _____ , as originally filed
pages _____ , filed with the demand
pages _____ , filed with the letter of _____

[ ] the sequence listing part of the description:
pages _____ , as originally filed
pages _____ , filed with the demand
pages _____ , filed with the letter of _____

2. With regard to the language, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is

[ ] the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

[ ] the language of publication of the international application(under Rule 48.3(b)).

[ ] the language of the translation furnished for the purposes of international preliminary examination(under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide** and/or amino acid sequence disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

[ ] contained inthe international application in written form.

[ ] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readable form

[ ] The statement that the subsequently furnished written sequence listing does not go beyond the disc losure in the international applicationas as filed has been furinshed.

[ ] The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. [ ] The amendments have resulted in the cancellation of:

[ ] the description, pages _____

[ ] the claims, Nos. _____

[ ] the drawings, sheet _____

5. [ ] This opinion has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box(Rule 70.2(c)).**

* *Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed." and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

** *Any replacement sheet containing such amendments must be referred to under item I and annexed to this report.*

INTERNATIONAL PRELIMINARY EXAMINATION

International aplication No.
PCT/KR00/00026

V. Reasoned statement under Article 35(2) with regard t novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	1-12	YES
	Claims		NO
Inventive step (IS)	Claims	1-12	YES
	Claims		NO
Industrial applicability (IA)	Claims	1-12	YES
	Claims		NO

2. Citations and explanations (Rule 70.7)

1) The following document have been considered for the purpose of this report:
D1= Microbiologia 1996, Sep. 12(3) pp359-370
D2 = Biotechnol Prog 1995, May-Jun. 11(3), pp288-293
D3 = Eur J Biochem, 1994 Jan 15, 219 (1-2) pp210-208

2) Novelty

Claims 1-5 relate to a recombinant enzyme with an improved D-amino acid oxidase activity. This invention, utilizes a D-amino acid oxidase which is fused with a bacterial hemoglobin (Vitreoscilla hemoglobin) gene to improve the yield of which can convert cephalosporin C into glutaryl-7-aminocephalosporanic acid (glutaryl-7ACA) in a bioreactor.

Document D1 discloses recombinant Acremonium chrysogenum strains for the industrial production of cephalosporin in a bioreactor. Document D1 discloses the concerns about the upper stream of the cephalosporin production pathway involving the Penicillin G, Penicillin N, deacetylcephalosporin (DAC), and Cephalosporin C which is produced by IPN acyltransferase(gene:penDE), IPN epimerase (gene:cefD), Expandase-hydroxylase(gene:cefEF), and DAC acetyltransferase (gene: cefG), respectively. Document D1 utilizes the recombinant Acremonium chrysogenum strains transformed with cefEF gene and expressing Expandase-hydroxylase. Additionally, the recombinant protein such as dao(D-amino acid oxidase) coupled with cephalosporin acylase or penDE(acyl-CoA:6-APA acetyltransferase) were employed. Document D1 discloses the introduction of foreign bacterial hemoglobin (Vitreoscilla hemoglobin) gene into Acremonium chrysogenum strains.

Since claims 1-5 in this invention discloses D-amino acid oxidase which is fused with a bacterial hemoglobin (Vitreoscilla hemoglobin) gene to improve the yield of which can convert cephalosporin C into glutaryl-7-aminocephalosporanic acid (glutaryl-7ACA), which is different from recombinant genes in D1 document, claims 1-5 are considered to be novel.

Document D2 discloses the promoter of hemoglobin gene, and D3 discloses intracellular expression of Vitreoscilla hemoglobin, not fused with catalytic enzyme, which is not so related to this invention as document D1.

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of:

3) Inventive Step

Claims 1-5 relate to a recombinant enzyme with an improved D-amino acid oxidase activity. This invention utilizes a D-amino acid oxidase which is fused with a bacterial hemoglobin (Vitreoscilla hemoglobin) gene to improve the yield of which can convert cephalosporin C into glutaryl-7-aminocephalosporanic acid (glutaryl-7ACA) in a bioreactor.

Document D1 utilizes the recombinant Acremonium chrysogenum strains transformed with cefEF gene into Acremonium chrysogenum strains for the industrial production of cephalosporin. Additionally, new biosynthetic capacities such as the production of 7-aminocephalosporanic acid (glutaryl-7ACA) or penicillin G have been achieved through the expression of the foreign genes dao(D-amino acid oxidase) coupled with cephalosporin acylase or penDE(acyl-CoA:6-APA acetyltransferase), respectively. Document D1 does disclose the introduction of foreign bacterial hemoglobin (Vitreoscilla hemoglobin) gene alone, not fused with product converting enzyme, into Acremonium chrysogenum strains for the increased production of the cephalosporin C by supplying enough oxygen.

Major difference between two inventions lies in the recombinant proteins utilizing D-amino acid oxidase which is fused with a bacterial hemoglobin (Vitreoscilla hemoglobin) gene in this invention, resulted in the improvements of the production of glutaryl-7-aminocephalosporanic acid (glutaryl-7 ACA), whereas D1 documents are utilizing dao(D-amino acid oxidase) coupled not with bacterial hemoglobin gene, but with cephalosporin acylase, which can improve the production of 7-aminocephalosporanic acid.

Utilizing D-amino acid oxidase which is fused with a bacterial hemoglobin (Vitreoscilla hemoglobin) gene is advantageous over D1 documents, since it can be helpful to introduce and control the enzyme converting Cephalosporin C into glutaryl-7 ACA and hemoglobin supplying oxygen in a close proximity. Usually, in the bioreactor, the converting enzyme is immobilized in the matrix for reuse. however, very poor conversion yields of product is obtained because oxygen molecules cannot be readily diffused in the matrixes. The expression of hemoglobin in the proximity to the catalytic enzyme acquired by D-amino acid oxidase which is fused with a bacterial hemoglobin (Vitreoscilla hemoglobin) gene is useful for the improvements product converting Cephalosporin C into glutaryl-7 ACA in a higher yield compare to the document D1.

Therefore, the subject-matter of claim 1-5 appear to involve an inventive step.

4) Industrial applicability

The subject matter of claim 1-5 is considered to be industrially applicable.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12N 15/53, 15/70, 15/62

A1

(11) International Publication Number: **WO 00/42197**

(43) International Publication Date: 20 July 2000 (20.07.00)

(21) International Application Number: PCT/KR00/00026

(22) International Filing Date: 14 January 2000 (14.01.00)

(30) Priority Data:
1999/865 14 January 1999 (14.01.99) KR
1999/5580 19 February 1999 (19.02.99) KR

(71) Applicant *(for all designated States except US)*: DAE SANG CORPORATION [KR/KR]; 52–1 Kayang–dong, Kangseo–ku, Seoul 157–200 (KR).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: KANG, Yong, Ho [KR/KR]; Yongnam University, Applied Microbiology Dept., 712–749 Dae–dong, Kyongsan–city, Kyongsangbuk–do (KR).

(74) Agent: HWANG, E–Nam; Yegun Building, 3rd floor, 823–42 Yoksam–dong, Kangnam–ku, Seoul 135–080 (KR).

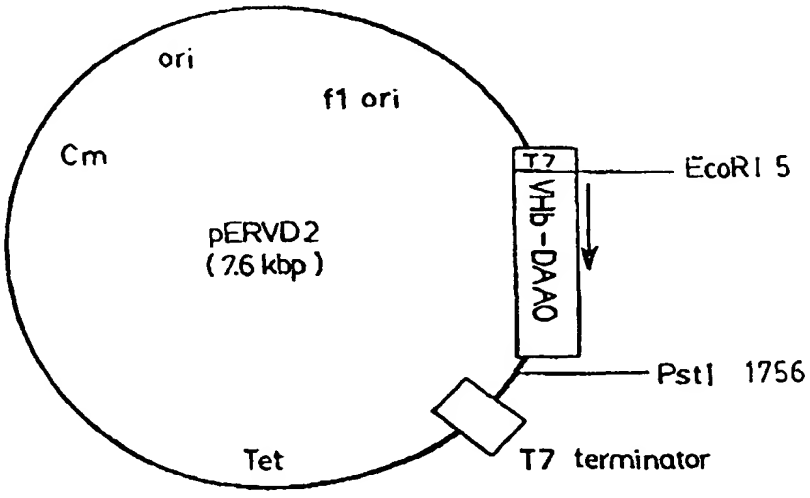
(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
In English translation (filed in Korean).

(54) Title: RECOMBINANT ENZYME WITH EXCELLENT D–AMINO ACID OXIDASE ACTIVITY AND PRODUCTION THEREOF

(57) Abstract

Disclosed is a recombinant enzyme which can convert cephalosprin C into glutaryl–7–aminocephalosporanic acid in a bioreactor at a high yield. A bacterial hemoglobin (*Vitreoscilla* hemoglobin) gene and a D–amino acid oxidase gene are fused to each other by PCR and the fused DNA fragment is cloned and expressed in *E. coli*. In a bioreactor, the recombiant enzyme VHb–DAAO can sufficiently supply oxygen as an electron acceptor by virtue of the fused hemoglobin, thereby showing an excellent capability of converting cephalosporin C.



Cm: Coding site of Choloramphenicol interfered gene

Tet: Coding site of Tetracycline interfered gene

T7: T7 RNA Polymerase promoter

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

RECOMBINANT ENZYME WITH EXCELLENT D-AMINO ACID OXIDASE ACTIVITY AND PRODUCTION THEREOF

TECHNICAL FIELD

The present invention relates to a recombinant enzyme with an improved D-amino acid oxidase activity. More particularly, the present invention relates to a D-amino acid oxidase which is fused with a bacterial hemoglobin and shows an excellent efficiency in converting cephalosporin C into glutaryl-7-aminocephalosporanic acid (glutaryl-7ACA) in a bioreactor. Also, the present invention is concerned with a method for producing such a recombinant enzyme.

BACKGROUND ART

With a share of as much as 40 % in the world market, semi-synthetic cephalosporin antibiotics are safer than other antibiotics and have antibacterial activity over a broad spectrum of bacteria. Usually, the chemical synthesis of semi-synthetic cephalosporin antibiotics is started from 7-aminocephalosporanic acid (7-ACA) which is conventionally prepared by chemically cleaving the aminoadipyl residue at position 7 in cephalosporin C that is purified from a microbial product.

The chemical processes including the cleavage of the aminoadipyl residue at position 7 inevitably produce pollution of the environment on account of toxic chemical reagents used and require a tremendous quantity of energy due to their ultra-low temperature reactions. In addition, there is international tendency toward the severe restriction of the organic solvent remaining in the final product. In result, there remains a need for developing processes which can substitute the chemical processes without producing pollution of the environment and allowing the toxic solvents to remain in the final product.

In this regard, bioprocesses have attracted intense attention. Particularly in preparing 7-aminocephalosporanic acid, advantage has been taken of enzymes of microbes. Such bioprocesses using enzymes of microbes are usually conducted in aqueous solution at room temperature and thus. require special

facilities in aspects of energy management and waste water treatment, enjoying the advantage of greatly reducing the production cost of 7-aminocephalosporanic acid.

Microbial conversion of cephalosporin C into 7-aminocephalosporanic acid is conducted in two enzymatic steps: cephalosporin C is oxidated into glutaryl-7ACA by D-amino acid oxidase and glutaryl-7ACA is cleaved at the bond between the glutaryl moiety and the 7-ACA moiety by glutaryl-7ACA acylase.

The D-amino acid oxidases obtained from eucaryotes including *Trigonopsis variabilis*, *Rhodotorula gracilis*, *Rhodotorula glutinis* and *Fusarium solani* have been used for the microbial conversion of cephalosporin C, thus far. The D-amino acid oxidases of such eucaryotes use FAD as a coenzyme. Thus, during their catalytic oxidation of cephalosporin C, oxygen atoms are always required as an electron acceptor. Since oxygen has extremely low solubility in water, a sufficiently large amount of oxygen must be continuously supplied to the bioreactor in order to achieve performance of the D-amino acid oxidase.

Most enzyme bioreactors employ matrixes in which enzymes are immobilized for reuse. When the D amino oxidases are immobilized in matrixes, however, very poor conversion yields of cephalosporin C are obtained because oxygen molecules cannot be readily diffused in the matrixes. In order to overcome this problem, the oxygen partial pressure in the bioreactor is raised. However, the oxygen pressure increase forces the bioreactor to be specially constructed in addition to being economically unfavorable owing to loss of a large quantity of oxygen.

DISCLOSURE OF THE INVENTION

Leading to the present invention, the intensive and thorough research on the bioconversion of cephalosporin C, repeated by the present inventor aiming to efficiently provide oxygen for immobilized D-amino acid oxidase resulted in the finding that, when an oxygen-carrying molecule was immobilized together with D-amino acid oxidase, the catalysis of the enzyme could be performed without a shortage of oxygen supply and that bacterial hemoglobin was effective as the oxygen-carrying molecule.

Therefore, it is an object of the present invention to provide a recombinant enzyme which shows stable and excellent amino acid oxidase activity when being

applied to a bioreactor for converting cephalosporin C into 7-aminocephalosporanic acid.

It is another object of the present invention to provide a method for producing such a recombinant enzyme.

Based on the present invention, the above objects could be accomplished by fusing a bacterial hemoglobin (*Vitroscilla* hemoglobin) gene and a D-amino acid oxidase gene to each other by a polymerase chain reaction to give a fusion gene, inserting the fusion gene in an expression vector, expressing the fusion gene in *E. coli*, purifying the fusion enzyme, and immobilizing the fusion enzyme in a polyacrylamide matrix to convert cephalosporin C.

BEST MODES FOR CARRYING OUT THE INVENTION

In the present invention, a bacterial hemoglobin gene, for example, *Vitreoscilla* hemoglobin (hereinafter referred to as "VHb") gene, is fused to a D-amino acid oxidase (hereinafter referred to as "D-AAO") by PCR. In this regard, a stretch of DNA in a 5' end region of the VHb gene is designed as a sense primer while a stretch of DNA in a 3' end region of the VHb gene is used as an antisense primer which has an overlapped portion with a stretch of DNA in a 5' end region of the D-AAO gene. Likewise, a sense primer for the amplification of the D-AAO gene is designed to have an overlapped portion with a stretch of DNA in 3' end region of the VHb gene. With respective primer sets, the VHb gene and the D-AAO gene are amplified. For fusion, these PCR products are mixed and re-amplified by use of a primer set consisting of the sense primer used to amplify the VHb gene and the antisense primer used to amplify the D-AAO gene. Alternatively, the VHb gene and D-AAO gene are mixed and may be fused by PCR in a DNA shuffling fashion without using primers.

Next, the VHb-DAAO fusion gene is introduced into an expression vector and expressed.

The catalytic activity of the recombinant enzyme can be measured by detecting the amount of H_2O_2, which is side-produced during the conversion of cephalosporin C into 7-aminocephalosporanic acid, in luminometric analysis.

To proceed with the research of the present invention, vector pUC8:16 carrying a VHb gene was granted from Professor Benjamin C. Stark, Illinois

Institute of Technology. After being deprived of its self promoter, the vector was amplified at the VHb gene region with reference to the reported gene sequence (Khosla and Bailey, 1988, Mol. Gen. Genet, 214:158-161; Dikshit and Webster, 1988: Gene 70:377-386).

As for the D-AAA gene used in the present invention, it was derived from *Trigonopsis variabilis* or *Rhodotorula gracilis*. These microogranisms were obtained from American Type Culture Collection: *Trigonopsis variabilis* ATCC10679 and *Rhodotorula gracilis* ATCC26217. From each of these microbes, genomic DNA was isolated, and used as a substrate to amplify a D-AAO gene (cDNA). For the cloning and the expressing of the D-AAO gene, commercially available vectors pALTER-EX2 (Promega, USA) and pKK223-3 (Pharmacia Biotech, Sweden) were utilized. PCR mixtures for the amplification of the genes of interest are given in Table 1, below.

TABLE 1

PCR Mixture Composition

	DNA	25mM $MgCl_2$	10X Buffer	DH_2O	2.5Mm dNTP	Taq polymerase	Primer
VHb	1μl	4μl	10μl	79μl	1μl	5 units	200pM
T. variabilis	2μl	4μl	10μl	79μl	1μl	5 units	200pM
R. gracilis	2μl	4μl	10μl	79μl	1μl	5 units	200pM

PCR was carried out in a thermal cycler, such as that manufactured by EquiBio, Belgium, identified as "ThermoJet", with 35 cycles of denaturing temperature at 94 °C for 1 min, annealing temperature at 55 °C for 1 min and extending temperature at 72 °C for 1 min, finally followed by 74 °C extension for an additional 4 min.

Taking advantage of the H_2O_2 side-produced during the bio-conversion of cephalosporin C, luminometry for analyzing the activity of the D-AAO used in the present invention is based on the following chemical reaction formulas:

D-AAO+Cephalosporin C+H_2O_2+O_2 →AKA-7ACA+NH_3+H_2O_2

$H_2O_2+2OH^-+Luminol+Peroxidase \rightarrow 4\text{-}Aminophthalate+N_2+2H_2O+Light$

This analytic method can determine the activity of the recombinant enzyme of the present invention very rapidly and accurately.

For the analysis of the recombinant enzyme, the recombinant vector of the present invention is introduced into *E. coli* which is, then, cultured in an LB broth. The cultured cells are harvested by centrifugation, washed with phosphate buffered saline (PBS, pH 7), and added with a solution containing cephalosporin C 20 mM, luminol 2 mM, peroxidase 1 unit, and FAD 5 μM. Using a luminometer (Tuner design, USA), the quantity of light emitted for 20 sec is measured. From this, the quantity of H_2O_2 is determined by use of a standard curve.

EXAMPLE 1

Fusion of VHb Gene and D-AAO Gene By PCR

In order to amplify a VHb gene, a stretch of DNA in a 5' end region of the VHb gene was designed as a sense primer while a stretch of DNA in a 3' end region of the VHb gene was used as an antisense primer which was so designed as to have an overlapped portion with a stretch of DNA in a 5' end region of the D-AAO gene. Likewise, a sense primer for the amplification of the D-AAO gene was designed to have an overlapped portion with a stretch of DNA in 3' end region of the VHb gene.

The DNA fragments thus amplified were purified and mixed with each other. In combination with the sense primer used to amplify the VHb gene and the antisense primer used to amplify the D-AAO gene, the amplified gene mixture was subjected to PCR with 35 cycles of denaturing temperature at 94 °C for 1 min, annealing temperature at 55 °C for 1 min and extending temperature at 72 °C for 1 min, finally followed by 74 °C extension for additional 4 min. The PCR composition used in this fusion PCR is given in Table 2, below.

TABLE 2

PCR Mixture Composition For VHb-DAAO Gene Fusion

	DNA	$MgCl_2$ (25mM)	10X buffer	dH_2O	dNTP (2.5mM)	Taq Polymerase	Primer
VHb	1μl	4μl	10μl	78μl	1μl	5 units	200pM
D-AAO	1μl	4μl	10μl	78μl	1μl	5 units	200pM

EXAMPLE 2

Fusion of VHb gene and D-AAO gene By DNA Shuffling

The VHb and the D-AAO DNA fragments amplified in Example 1 were purified and mixed with each other. The mixture was subjected to PCR without primers. The PCR is carried out with 35 cycles of denaturing temperature at 94 °C for 1 min, annealing temperature at 55 °C for 1 min and extending temperature at 72 °C for 1 min, finally followed by 74 °C extension for additional 4 min. The PCR composition in this fusion PCR is given in Table 3, below.

TABLE 3

PCR Mixture Composition For VHb-DAAO Gene Fusion

	DNA	$MgCl_2$	10X Buffer	dH_2O	DNTP (2.5mM)	Taq polymerase
VHb	10μl	4μl	10μl	64μl	1μl	5 units
D-AAO	10μl	4μl	10μl	64μl	1μl	5 units

EXAMPLE 3

Cloning of VHb-DAAO Fusion Gene

To produce blunt ends, VHb-DAAO fusion DNA fragments amplified in Examples 1 and 2 were treated with Klenow enzyme at 25 °C for 30 min in a Klenow mixture containing a Klenow fragment 4 units, dNTP (2.5 mM) 3 μl, and

10x buffer 3 μl. The blunt-ended fusion DNA fragments were purified by ethanol precipitation, and sub-cloned in expression vectors, respectively.

For the sub-cloning, pALTER-Ex2 and pKK223-3 were linearlized at *Stu*I and *Sma*I, respectively, and dephosphorylated with alkaline phosphatase, followed by incubation for 1 hour at 16 °C along with the fusion gene fragment and T4 DNA ligase.

EXAMPLE 4

Bio-Conversion of Cephalosporin C in Packed Bed Bioreactor

E. coli was transformed with the vectors constructed in Example 3, and cultured overnight in LB broth to obtain cell extracts. These cell extracts were precipitated by ammonium sulfate and subjected to dialysis, followed by passing the dialysates through anionic exchange resins (DEAE-Sephadex FF) to purify D-AAO and VHb-DAAO, respectively. These purified enzymes were immobilized in polyacrylamide matrixes which were then cut into cubes (1.5 x 1.5 x 1.5 mm) and put in packed bed bioreactors (1.5 cm in diameter, 15 cm in length).

20 mM cephalosporin C in Tris-HCl buffer (pH 8) was circulated at a flow rate of 1.5 mL/min through the packed bed bioreactors with the aid of a peristaltic pump while oxygen was continuously supplied to the batch type vessels. At regular time intervals, samples were taken from the reactors and quantitatively measured for the H_2O_2 produced as a result of the bioconversion of cephalosporin C. The results are given in Table 4, below. As indicated in Table 4, the by-product H_2O_2 was hardly produced in the D-AAO immobilized reactor because of the oxygen deficiency resulting from the resistance of the matrix to oxygen diffusion while the VHb-DAAO fusion enzyme immobilized reactor allowed H_2O_2 to be produced at an amount 12 times as much as that of the D-AAO immobilized reactor within 45 min. Therefore, the VHb-DAAO fusion enzyme of the present invention could effectively perform the conversion of cephalosporin C without increasing the oxygen partial pressure in the reactor.

The novel recombinant *E. coli*, which was transformed with the recombinant vector pALTER-Ex2 carrying the VHb-DAAO fusion gene of the present invention, was deposited in the Korean Collection for Type Culture at

Korea Research Institute of Bioscience and Biotechnology (KRIBB) under the deposition No. KCTC 8923P on Jan. 18, 1999.

TABLE 4

Conversion Ability of Recombinant D-Amino Acid Oxidases in Terms of Production of H_2O_2

Time Period (min)		0	15	30	45	60	90	120
$H_2O_2(\mu M)$	D-AAO	0	0.5	0.5	0.8	1.0	1.0	1.0
	VHb-DAAO	0	2.0	4.5	12.0	12.0	12.0	12.0

INDUSTRIAL APPLICABILITY

As elucidated in the above examples, the recombinant enzyme VHb-DAAO can be obtained from the novel recombinant E. coli, which harbors a fusion gene consisting of a *Vitreoscilla* hemoglobin gene and a D-amino acid oxidase and can be applied to a bioreactor which can industrially convert cephalosporin C into glutaryl-7ACA.

INDICATIONS RELATING TO DEPOSITED MICROORGANISM AND OTHER BIOLOGICAL MATERIALS

A. The indications made below relate to the deposited microorganism and other biological materials referred to in the description on Page 9, Lines 14 – 16	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☐	
Name of depositary institution (*including postal code and country*): The Korean Collection for Type Cultures (KCTC) at the Korea Research Institute of Bioscience and Biotechnology #52, Oun-Dong, Yusong-Gu, Taejon, 305-333, Korea	
Date of deposit January 18, 1999	Accession Number KCTC 8923P
C. ADDITIONAL INDICATIONS*(leave blank if not applicable)*: This information is continued on an additional sheet ☐	
D. CHARACTERISTICS FOR WHICH INDICATIONS ARE MADE	
E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*	
The indications listed below will be submitted to the International Bureau later	

For receiving Office use only	For International Bureau use only
☐ This sheet was received with the international application	☐ This sheet was received by the International Bureau on
Authorized officer	Authorized officer

CLAIMS

1. A recombinant fusion enzyme VHb-DAAO, expressible from a recombinant fusion gene consisting of a gene encoding a bacterial hemoglobin and a gene encoding a D-amino acid oxidase.

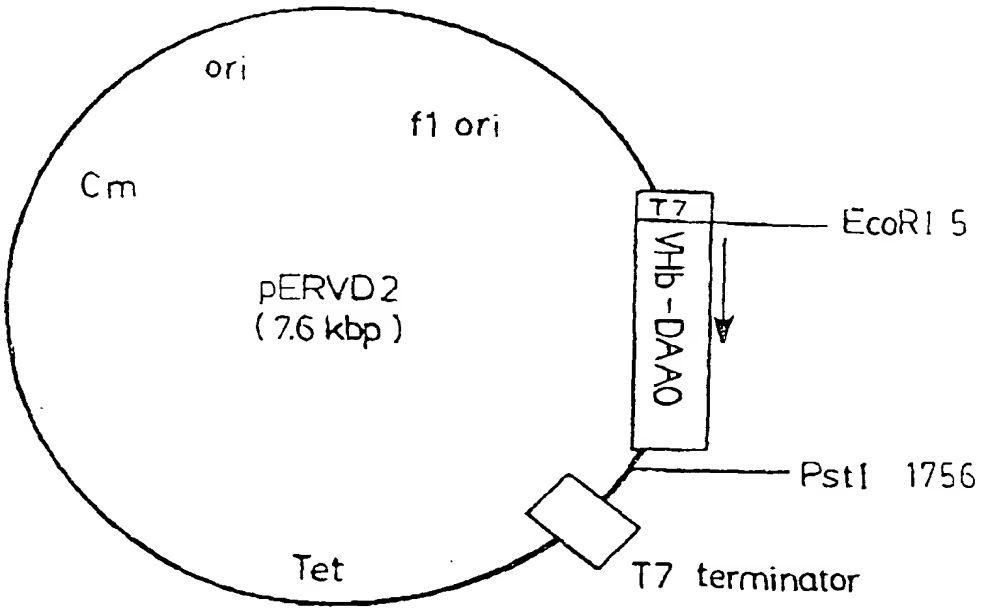
2. A recombinant fusion enzyme bacterial hemoglobin-D-amino acid oxidase as set forth in claim 1, wherein said bacterial hemoglobin contains a full or a partial length of a *Vitreoscilla* hemoglobin peptide sequence or its functionally analogous peptide sequence.

3. A method for producing a recombinant fusion enzyme VHb-DAAO, which comprises fusing a bacterial hemoglobin gene and a D-amino acid oxidase gene to each other by a polymerase chain reaction to give a fusion gene, inserting the fusion gene in an expression vector, expressing the fusion gene in *E. coli*, and purifying the fused enzyme VHb-DAAO.

4. A recombinant vector pALTER-EX2/VHb-DAAO, which is constructed by introducing a fusion gene consisting of a bacterial hemoglobin gene and a D-amino acid oxidase.

5. A recombinant E. coli (KCTC 8923P), which is transformed with the recombinant vector pALTER-EX2/VHb-DAAO of claim 4.

FIG. 1



Cm: Coding site of Choloramphenicol interfered gene

Tet: Coding site of Tetracycline interfered gene

T7: T7 RNA Polymerase promoter

THIS PAGE BLANK (USPTO)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR00/00026

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 C12N 15/53, C12N 15/70, C12N 15/62

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimun documentation searched (classification system followed by classification symbols)

IPC7 C12N 15/53, C12N 15/70, C12N 15/62

Documentation searched other than minimun documentation to the extent that such documents are included in the fileds searched

Electronic data base consulted during the intertnational search (name of data base and, where practicable, search trerms used)
PubMed, "Vhb, cephalosporin, dAO, gene, 'D amino acid oxidase', ACA, vitreoscilla, hemoglobin"

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Microbiologia 1996 Sep ; 12(3) : pages 359-370 Diez B, Mellado E, Fouces R, Rodriguez M, Barredo JL Recombinant Acremonium chrydogenum strains for the industrial production of cephalosporin	1 - 5
A	Biotechnol Prog 1995 May-Jun ; 11(3) : pages 288 - 293 Tsai PS, Kallio PT, Bailey JE Fnr, a global transcriptional regulator of Escherichia coli, activates the Vitreoscilla hemoglobin (Vhb) promoter and intracellular Vhb Expression increases cytochrome d promoter activity	1 - 5
A	Eur J Biochem 1994 Jan 15 ; 219(1-2) : pages 210 - 208 Kallio PT, Kim DJ, Tsai PS Intracellular expression of Vitreoscilla hemoglobin alters Escherichoa coli energy metabolism under oxygen-limited conditions	1 - 5

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevence
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevence; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevence; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 APRIL 2000 (29.04.2000)	02 MAY 2000 (02.05.2000)

Name and mailing address of the ISA/KR	Authorized officer
Korean Industrial Property Office Government Complex-Taejon, Dunsan-dong, So-ku, Taejon Metropolitan City 302-701, Republic of Korea	KIM, Hycong Joon
Facsimile No. 82-42-472-7140	Telephone No. 82-42-481-5589

Form PCT/ISA/210 (second sheet) (July 1998)

THIS PAGE BLANK (USPTO)